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14. ABSTRACT

The purpose of this project is to understand how metabolic factors regulate the development of post-traumatic osteoarthritis. Specifically, the goals of this project are to test the idea that alterations in systemic inflammation caused by changes in the gut microbiota promote the occurrence of post-traumatic osteoarthritis. The project examines the development of osteoarthritis following a single overload to the knee joint under conditions of altered gut microbiota caused by genetic background or chronic antibiotic treatment. In the first year of this project we have made considerable progress, receiving approval from institutional animal care and use ethical reviews, performing animal breeding and mechanical stimulations known to cause the development of osteoarthritis in control groups. At this time we have completed all live animal experimentation in the project and have started data analysis. Early results from the project suggest that alterations in the gut microbiota are causing changes in bone tissue mechanical properties that may mediate the effects of mechanical overloads on subsequent cartilage degeneration.

15. SUBJECT TERMS
post-traumatic osteoarthritis; osteoarthritis; microbiome; obesity; systemic inflammation

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1. INTRODUCTION

Osteoarthritis is characterized by degradation of joint cartilage and alterations in peri-articular bone leading to pain and loss of mobility. Osteoarthritis that develops after a single traumatic event such as a fall or fracture near the join is classified as post-traumatic osteoarthritis. Excessive joint loads are recognized as the primary contributor to the development of post-traumatic osteoarthritis and other forms of osteoarthritis. Recent findings suggest that low-grade chronic systemic inflammation can aggravate the development of osteoarthritis. In this 18-month long Discovery award we test the concept that low-grade chronic systemic inflammation caused by changes in the gut microbiota regulate the development of post-traumatic osteoarthritis. To test this idea, we use a mouse model in which a short period of mechanical loading, applied to the joint, leads to cartilage degeneration and apply the technique to mice with varying amounts of low-grade chronic systemic inflammation associated with alterations in the gut microbiota.

2. KEYWORDS

osteoarthritis
post-traumatic osteoarthritis
mechanical loads
microbiome
systemic inflammation

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Specific Aim	Timeline	Site 1	Completion Date
Aim	Months		
Animal Breeding to Achieve Desired Sample Size	5-10	Dr. van der Meulen	05/01/2016
Pre-treatment of Animal Groups (antibiotic treatment, etc.)	6-11	Dr. Hernandez	09/01/2016
Mechanical Loading	10-15	Dr. Hernandez Dr. van der Meulen	09/01/2016
Micro-CT and Histology	11-18	Dr. Hernandez Dr. van der Meulen	25% Complete
Serum Assays	11-18	Dr. van der Meulen Dr. Hernandez	
Manuscript Preparation	17-18	Dr. Hernandez Dr. van der Meulen	
Local IRB/IACUC Approval	1	Dr. Hernandez	05/07/2015
Milestone Achieved: HRPO/ACURO Approval	2-5	Dr. Hernandez	11/03/2015

- **What was accomplished under these goals?**

1) Major activities
Four groups of animals were bred in our facility and raised to 20 weeks of age. Animals in two groups received treatment starting at weaning (4 weeks of age). Treatment included either a high fat diet (34% fat

content, D12492, Research Diets Inc.) or chronic oral antibiotics in drinking water (1.0 g/L ampicillin and 0.5g/L neomycin). The antibiotics were chosen as they have poor bioavailability and therefore have minor extraintestinal effects.

STUDY GROUP	Inflammation	Body Weight
TLR5KO	Mild	Mild Increase
TLR5KO+Antibiotic	Normal	Normal
WT (negative control)	Normal	Normal
WT+High Fat Diet (positive control)	Moderate	Moderate Increase

At 20 weeks of age animals were anesthesized and submitted to a single bout of tibial loading (1,200 cycles at 4Hz, 5 minutes of exposure). Loading was applied with a maximum load of 9N, 6N or 4.5 N. The 9N load has been shown to cause cartilage degeneration at 2 weeks after loading.
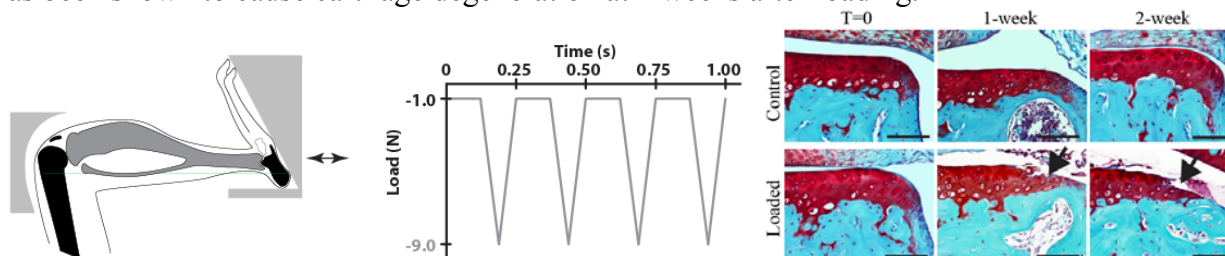
Figure 1. An illustration of the loading modality, applied load waveform and expected cartilage degeneration in the WT (negative control) group.

2) Specific Objectives
The objectives of this project were to determine the effects of low-grade systemic inflammation on cartilage degeneration caused by a single bout of mechanical loading. Our study groups (see part 1) include two methods of increasing systemic inflammation, the TLR5KO mouse (which spontaneously develops low grade systemic inflammation due to its microbiota) and the TLR5KO mouse treated with antibiotics to prevent development of systemic inflammation. Wild type mice are included as control groups.

3) Significant Results
Animals were raised in our facility and submitted to tibial loading. Tissue has been collected and is currently undergoing analysis.

4) Other achievements
   In addition to the proposed work we also wrote and published a review article on the general topic of the microbiome and musculoskeletal disease and completed a pilot study to evaluate the bone phenotype in the experimental groups.

Discussion of Goals not Met
We have met all goals for this reporting period.

What opportunities for training and professional development has the project provided?

▪ **Training**
The following graduate students participated in one on one training with a mentor (the PI):
Jason D. Guss, M.S.
Marysol Luna, B.S.

The following undergraduate students received one on one training from mentors (the PI and participating graduate students):
Adrian Alepuz

Gabriel Guisado
Taylor Sandoval
Laura Vasquez-Bolanos

- **Professional Development**

Conference Attendance/Workshop Participation:
Gordon Research Conference in Musculoskeletal Biology & Bioengineering (08/07/16-08/12/16)
Christopher J. Hernandez, Ph.D. (PI)

Society of Hispanic Professional Engineers (11/11/16-11/14/16)
Graduate Institute (training for graduate students)
Marysol Luna, B.S.

How were the results disseminated to communities of interest?

- **Outreach Activities**
Dr. Hernandez contributed to the following programs working to enhance public understanding and increasing interest in learnd and careers in science, technology and the humanities:

Society of Hispanic Professional Engineers (11/11/16-11/14/16)
Dr. Hernandez Chaired the Student Scientific Sessions
Dr. Hernandez served as a panelist to the Graduate Institute (professional preparation for graduate students) and the Faculty Institute (professional preparation for junior faculty)

- **What do you plan to do during the next reporting period to accomplish the goals?**
In the final 6 months of the project we will complete histology and serum assays and prepare a manuscript describing the findings for submission to a peer-reviewed journal.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**
Nothing to Report (final results on for the principal discipline are pending)

- **What was the impact on other disciplines?**
    We performed a pilot experiment to validate our methodology prior to the primary experiment. In this pilot work we examined the bone phenotype of the TLR5KO mice and the wild type control mice under normal conditions and in conditions of altered gut microbiota caused by chronic treatment with oral antibiotics. We found that antibiotic treatment altered the contents of the gut flora in a way that resulted in changes in bone tissue mechanical properties. Our findings demonstrate that alterations in the gut microbiota can change bone tissue mechanical properties in ways that reduce bone strength without changing bone shape, size or bone mineral density. This early finding suggests that alterations in the gut microbiota may explain situations in which risk of fragility fracture is greater than expected from clinical BMD scans and supports the idea that evaluation of the gut flora may be relevant to screening for risk of osteoporosis-related fracture.

- **What was the impact on technology transfer?**
Nothing to Report

- **What was the impact on society beyond science and technology?**
Nothing to Report

5. CHALLENGES/PROBLEMS

▪ **Changes in approach and reasons for change**
Based on recent reports of osteoarthritis generated by a single loading event (Ko et al. 2016) we have adjusted the proposed study groups in order to address our hypothesis and research questions. The new study groups include three different load magnitudes (4.5 N, 6 N, 9N) during load application and joints from all animals are examined 2 weeks after applied loading. The approach allows us to address our primary hypothesis by determining if osteoarthritis is generated by a more modest load magnitude in animals with altered systemic inflammation.

▪ **Actual or anticipated problems or delays and actions or plans to resolve them**
> Nothing to Report

▪ **Changes that had a significant impact on expenditures**
> Nothing to Report

▪ **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
> Nothing to Report

▪ **Significant changes in use or care of human subjects**
> Not Applicable

▪ **Significant changes in use or care of vertebrate animals.**
> Nothing to Report

▪ **Significant changes in use of biohazards and/or select agents**
> Nothing to Report

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications

- Hernandez, C.J., Guss, J.D., Luna, M., Goldring, S.R. (2016) "Links Between the Microbiome and Bone" J Bone Miner Res. 31(9): 1638-46. doi: 10.1002/jbmr.2887
  > Status of Publication: Published
  > Acknowledgement of federal support: Yes

- Guss, J.D., Horsfield, M.W., Fontenele, F.F., Sandoval, T.N., Apoorva, F., Lima, S.F., Bicalho, R.C., van der Meulen, M.C.H., Singh, A., Goldring, S.R., Hernandez, C.J. (2016) "The Gut Microbiome Influences Bone Strength and Regulates Differences in Bone Biomechanical Phenotype Among Inbred Mouse Strains"
  > Status of Publication: Other (in preparation)
  > Acknowledgement of federal support: Yes

Books or other non-periodical, one-time publications.
Nothing to Report

Other publications, conference papers, and presentations.

- Guss, J.D., Horsfield, M.W., Fontenele, F.F., Sandoval, T.N., Apoorva, F., Lima, S.F., Bicalho, R.C., van der Meulen, M.C.H., Singh, A., Goldring, S.R., **Hernandez, C.J.** (2016) "The Gut Microbiome Influences Bone Strength and Regulates Differences in Bone Biomechanical Phenotype Among Inbred Mouse Strains" 42nd Annual Meeting of the American Society for Bone and Mineral Research, Atlanta, GA, USA.

Status of Publication: Published
Acknowledgement of federal support: Yes

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
Nothing to Report

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Christopher J. Hernandez, Ph.D.*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0002-0712-6533
Nearest person month worked:	*1.0*
Contribution to Project:	*Dr. Hernandez is the PI for this project and has overseen all experimental work and data analysis.*

Name:	*Marjolein M.C. van der Meulen, Ph.D.*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0
Contribution to Project:	*Dr. van der Meulen has worked to oversee experimental procedures on the experimental animals and in data analysis.*

Name:	*Jason D. Guss, M.S.*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.5
Contribution to Project:	*Mr. Guss has been directly involved in breeding the mice for the proposed work, establishing experimental methods and performing experiments and analyzing data.*

Name:	*Marysol Luna, B.S.*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5.0
Contribution to Project:	*Ms. Luna has been led animal breeding and experimental manipulations of the animals and coordinated final data acquisition and data analysis.*
Funding Support:	*Sloan Fellowship*

Name:	Erin N. Cresswell
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.5
Contribution to Project:	Ms. Cresswell contributed to animal loading and analysis of images from the animals.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. Dr. Ley has already completed her contribution to the project regarding breeding and handing of the TLR5KO mouse and will not be participating in the last 6 months of the project (she has moved to a foreign institution). All other participants are contributing as established.

Dr. Goldring is still participating in the project but his effort is less than 1 month and he is therefore not listed above.

The following changes in other research support for Dr. van der Meulen are shown below. Dr. van der Meulen
NSF 1636012   van der Meulen (PI)   9/1/16-8/31/19
                      Total Costs: $300,000   Effort: 0.5
Title "Mechanobiology of Cortical and Cancellous Bone Adaptation"

NSF 1605935   van der Meulen (PI)   7/1/16-6/30/19
                      Total Costs $300,000   Effort: 0.5
Title "Genetic Control of Bone Structure"

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
Not Applicable

QUAD CHARTS:
Not Applicable

9. APPENDICES
The following items are in the appendix:

Hernandez, C.J., Guss, J.D., Luna, M., Goldring, S.R. (2016) "Links Between the Microbiome and Bone" J Bone Miner Res. 31(9): 1638-46. doi: 10.1002/jbmr.2887

Guss, J.D., Horsfield, M.W., Fontenele, F.F., Sandoval, T.N., Apoorva, F., Lima, S.F., Bicalho, R.C., van der Meulen, M.C.H., Singh, A., Goldring, S.R., Hernandez, C.J. (2016) "The Gut Microbiome Influences Bone Strength and Regulates Differences in Bone Biomechanical Phenotype Among Inbred Mouse Strains" 42nd Annual Meeting of the American Society for Bone and Mineral Research, Atlanta, GA, USA.

Links Between the Microbiome and Bone

Christopher J Hernandez,[1,2,3] Jason D Guss,[2] Marysol Luna,[1] and Steven R Goldring[3]

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[3]Hospital for Special Surgery, New York, NY, USA

ABSTRACT

The human microbiome has been shown to influence a number of chronic conditions associated with impaired bone mass and bone quality, including obesity, diabetes, and inflammatory bowel disease. The connection between the microbiome and bone health, however, has not been well studied. The few studies available demonstrate that the microbiome can have a large effect on bone remodeling and bone mass. The gut microbiome is the largest reservoir of microbial organisms in the body and consists of more than a thousand different species interacting with one another in a stable, dynamic equilibrium. How the microbiome can affect organs distant from the gut is not well understood but is believed to occur through regulation of nutrition, regulation of the immune system, and/or translocation of bacterial products across the gut endothelial barrier. Here we review each of these mechanisms and discuss their potential effect on bone remodeling and bone mass. We discuss how preclinical studies of bone-microbiome interactions are challenging because the microbiome is sensitive to genetic background, housing environment, and vendor source. Additionally, although the microbiome exhibits a robust response to external stimuli, it rapidly returns to its original steady state after a disturbance, making it difficult to sustain controlled changes in the microbiome over time periods required to detect alterations in bone remodeling, mass, or structure. Despite these challenges, an understanding of the mechanisms by which the gut microbiome affects bone has the potential to provide insights into the dissociation between fracture risk and bone mineral density in patients including those with obesity, diabetes, or inflammatory bowel disease. In addition, alteration of the gut microbiome has the potential to serve as a biomarker of bone metabolic activity as well as a target for therapies to improve bone structure and quality using pharmaceutical agents or pre- or probiotics. © 2016 American Society for Bone and Mineral Research.

KEY WORDS: MICROBIOME; INFLAMMATION; OSTEOPOROSIS; FRACTURE; OSTEOIMMUNOLOGY

Introduction

The human microbiome consists of the microbial species that inhabit the human body and their secreted products.[1,2] Each individual hosts trillions of microbes, a population that vastly outnumbers native mammalian cells. Microbiota confer benefits to the host that include vitamin production,[3] nutrient and energy extraction from diet,[4] metabolic function,[5] regulation of innate and adaptive immunity,[6,7] and protection from pathogenic organisms.[8] Alterations in the microbiome have been associated with a number of chronic conditions in humans, including inflammatory bowel diseases,[9] obesity,[10] metabolic disease,[11] malnutrition,[12] neurological disorders,[13] cancer,[14] and cardiovascular disease.[15]

The human microbiome is established soon after birth, usually by colonization by microbial flora present in the birth canal.[16] The microbiota is shaped subsequently by diet and environmental exposure and reaches a steady state at about 3 years of age.[16] The great majority of the human microbiome is located within the gastrointestinal system. The human gut microbiota consists of more than 1000 distinct microbial species, many of them not yet well characterized. Roughly two-thirds of the microbial species composition is unique to each individual.[17]

The human gut microbiota is dominated by organisms from the *Bacteroidetes* and *Firmictues* phyla.[18] Once established in an individual, the contents of the microbial community in the gut enter a dynamic equilibrium as the hundreds of different species compete and interact with one another and the host immune system in complex networks of interdependence. The relative abundance of species within the gut flora fluctuates from day to day based on changes in diet,[18,19] but in general retains its basal constitutive state despite these transient disruptions. For example, after a stimulus such as a course of antibiotics or short gastrointestinal infection, the contents of the gut microbiota mostly return to their initial state, although the resulting gut microbial community may be less stable than it was before treatment[20] and small changes in content may occur (eg, species with similar function may replace each other[19]). Hence, although the gut microbiome is relatively stable, it can be changed by long periods of sustained stimuli or factors that produce large perturbations in the gut flora. Factors that have been shown to alter the steady state of the gut flora include aging,[16] diet,[19] environment,[21] physiologic state, and chronic treatment with oral antibiotics.[22]

Alterations in the composition of the gut microbiome have been implicated either directly or indirectly in the deregulated

bone remodeling associated with obesity, diabetes, inflammatory bowel disease, and rheumatoid arthritis (Table 1). In this review, we explore the potential effects of the gut microbiome on bone, first by discussing the potential mechanisms that explain how changes in microbial populations in the gut can have effects at distant organs, and second by reviewing preclinical findings linking changes in the gut microbiome to alterations in bone mass. Lastly, we discuss the challenges in the study of bone-microbiome interactions.

How the Gut Microbiome Affects Distant Organs

Although there are a number of studies demonstrating that the gut microbiome can influence the natural history of many clinical disorders, the field has been limited in terms of mechanistic explanations,[23] especially with regard to the effect of the microbiome on organs distant from the gut. Fig. 1 illustrates the three potential mechanisms by which the gut microbiota can influence bone tissues: regulation of nutrient absorption at the gut epithelium, regulation of the mucosal and systemic immune system, and translocation of microbial contents across the gut endothelial barrier.

It is well established that alterations in the gut microbiome can alter nutrient absorption, including the ability of the host to absorb calories from food. For example, low-dose antibiotics commonly used in livestock feed promote increased animal growth and bone size by altering the gut flora and increasing caloric absorption from food.[24–26] In addition to influencing caloric absorption, the intestinal microbiota aids in host and microbial metabolism through the biosynthesis of vitamins, including cobalamin (B12), biotin (B7), folate, thiamine (B1), pyridoxal phosphate, pantothenic acid (B5), niacin (B3), vitamin K, and tetrahydrofolate.[6,16] These vitamins are absorbed at the gut lining and distributed throughout the body through the systemic circulation along with nutrients. Vitamins metabolized in the gut have functions throughout the body, including regulation of the metabolism of proteins, aiding in the formation of red blood cells, maintenance of the central nervous system, metabolism of carbohydrates and fat, regulation of cell division and repair, ensuring proper cardiac function, regulation of blood clotting, and maintenance of bone mass.[16]

The gut microbiome is also known to influence the development and function of the host immune system. The immune system is stimulated at the gut endothelial barrier by metabolites released by the gut flora as well as by direct contact between microorganisms and immune cells.[27] The interactions between the gut flora and the immune system are reciprocal; the immune system regulates commensal composition and localization, while the interactions with the commensal flora are crucial for the development and function of an effective immune system.[27] Immune cells, including T cells and dendritic cells, interact with the microbial flora at the gut lining and migrate to lymph nodes to activate either pro- or anti-inflammatory immune responses. These cells also may release soluble pro- or anti-inflammatory mediators or cytokines into the circulation and by this mechanism modulate systemic bone remodeling. Additionally, activated immune cells can migrate to the bone tissues where they can directly regulate bone remodeling by the release of products, including the potent osteoclast-inducing factor, receptor activator of NF-κβ ligand (RANKL), or other bone active molecules.[28,29] Bacteria-derived short-chain fatty acids are well-known regulators of immune cells. They are synthesized by bacterial fermentation of carbohydrates in the colon where they can act as an energy source for epithelial cells in the colon but can also promote the induction and activity of regulatory T cells and thereby inhibit immune cell responses.[27,30–32] The commensal flora also compete with invading organisms for nutrients and produce antimicrobial molecules and metabolites that hinder pathogen survival and promote tighter junctions between epithelial cells to prevent translocation of pathogens into the systemic circulation. Lastly, the gut microbiome plays a crucial role in immune system development and control by regulating and suppressing inflammatory responses to food products that can serve as ingested antigens.[27,33–36]

The gut microbiome can also influence distant organs by introducing microbial-associated molecular patterns (MAMPs) into the systemic circulation.[37] MAMPs such as lipopolysaccharide, peptidoglycan, flagellin, and cell-free DNA secreted by bacteria or which are retained after cell death are sufficiently small enough to be transported across the gut endothelial barrier and enter into the systemic circulation. Once distributed to remote organs such as bone, MAMPs can activate innate or adaptive immune responses to produce local inflammation. In bone, MAMPs are known to have a direct effect on bone remodeling through stimulation of innate immune receptors on bone cells, including toll-like receptor 2 (TLR2) (which responds to peptidoglycan),[38] TLR4 (which responds to lipopolysaccharide),[39,40] and TLR5 (which responds to flagellin).[41–43] In addition to the translocation of MAMPs, viable bacteria can cross the gut endothelial barrier through a process known as bacterial

Table 1. Alterations in the Gut Microbiota Have Been Associated With Many of the Factors That Cause Osteoporosis and/or Fragility Fractures

Contributor to osteoporosis	Reported alterations in gut microbiota
Poor acquisition of bone mass during growth leading to low BMD in adulthood	Absence of gut microbiota associated with altered bone mass in mice[57,59]
Alterations in circulating sex hormones	Chemically induced estrogen depletion does not result in bone loss in germ-free animals[58]
	Probiotic treatment reduces ovariectomy-associated bone loss[58,70]
Diet/nutrition	Gut microbiota regulate production/absorption of vitamins[6]
Aging	Gut microbiota composition is correlated with indices of frailty in the elderly (Barthel index, functional independence measures)[52,96]
Obesity/diabetes	Gut microbiota influence caloric intake and the development of obesity[10,65]
Gastrointestinal disease	Inflammatory bowel disease is related to the microbiome and leads to osteopenia independent of its effects on nutrition[10,83,97]
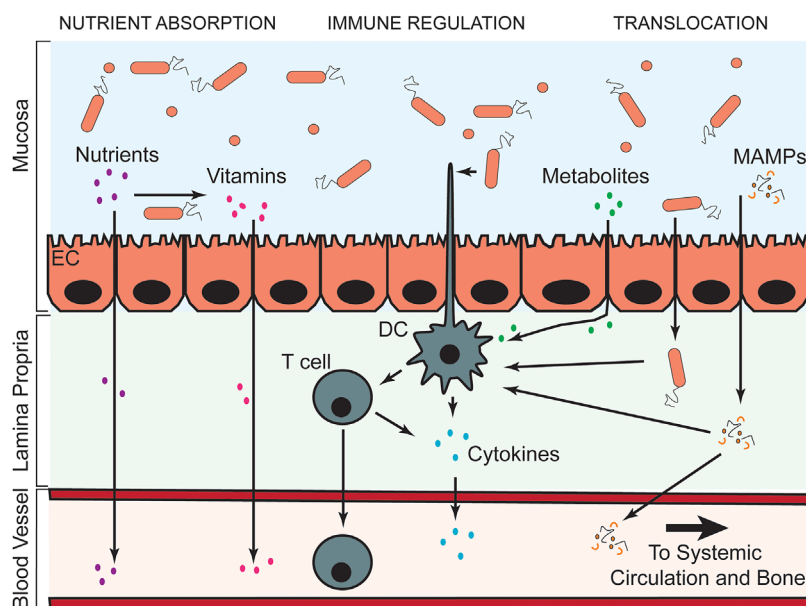
Fig. 1. The gut microbiome can influence remote organs through regulation of nutrient absorption, regulation of the immune system, or translocation of bacterial products. Molecular products and activated T cells enter the systemic circulation, where they can migrate to distant organs including bone. DC = dendritic cell; EC = endothelial cell; MAMPs = microbial-associated molecular patterns.

translocation. Once considered a controversial topic, bacterial translocation is now a well-recognized phenomenon.[44,45] In individuals with a normal immune system, bacterial translocation is rare, but in disease states, gut inflammation can increase intestinal permeability allowing more bacteria to cross the endothelial barrier.[46] Translocation of gastrointestinal bacteria has been detected in patients with bowel cancer, bowel obstruction, Crohn's disease, ulcerative colitis, hemorrhagic shock, and trauma.[46] Translocated bacteria are usually killed rapidly by immune cells, but even after induction of cell death, small amounts of MAMPs may be released into the systemic circulation.[37] Lastly, some bacteria that cross the gut endothelial barrier can penetrate and survive inside native cells where they avoid an immune response.[37,47] It is unclear if translocated bacteria are able to migrate to distant organs (presumably while occupying a host cell), although evidence that orthopedic implant infection can start from "hematogenous seeding" demonstrates that bacteria may travel to bone through the circulatory system.[48]

Evidence of Bone-Microbiome Interactions

There is no single cause of osteoporosis, and multiple mechanisms are involved in the pathogenesis of osteopenia, including, for example, poor acquisition of bone mass during skeletal growth, limited physical activity, poor nutritional history, alterations in sex steroid hormone levels, and genetic background. Additionally, osteopenia can be secondary to other conditions, including inflammatory disorders such as inflammatory bowel disease or pharmacological treatments such as glucocorticoids.[49,50] To date, there is relatively little direct evidence relating osteoporosis or osteopenia to the state of the gut microbiome, although there is substantial indirect evidence. For example, gut microbial diversity changes with age[51] and is

negatively correlated with clinical indices of frailty in the elderly.[52] Additionally, many of the risk factors associated with the development of osteoporosis and osteopenia are also associated with alterations in the gut microbiome (Table 1).

Bone growth

Recent investigations in animal models show that the presence and contents of the gut microbiota influence the accumulation of bone mass during growth. One of the most useful tools for studying the gut microbiome are germ-free mice. Germ-free animals are raised in a sterile incubator and are never exposed to detectable microorganisms and, therefore, do not have a microbiome (germ-free incubators are not to be confused with specific pathogen-free facilities).[2,53,54] Additionally, animals raised in a germ-free environment fail to develop a mature immune system and display altered physiology and organ morphology,[55] which is a recognized limitation of their use, but at the same time they provide one of the best means of studying the microbiome.[56] There are conflicting data regarding the effects of a germ-free state on bone. An early report showed that germ-free 7- to 9-week-old female C57Bl/6 mice had increased bone mineral density, 39% greater femoral metaphyseal trabecular bone volume fraction, reduced osteoclast surface, and increased mineralizing surface compared with conventionally raised animals.[57] The bone phenotype in germ-free mice was shown to be reversible by reconstituting the gut microbiota with flora from a conventionally raised animal. Partially confirming this finding, a recent study reported that female germ-free C57Bl/6 mice had greater femoral cortical volume and cortical thickness than conventionally raised mice at 20 weeks of age, although there was no significant change in trabecular bone volume fraction.[58] In contrast, a study of 8-week-old male BALB/c mice reported that germ-free animals had reduced femoral length, cortical thickness, and bone mineral density

compared with animals raised in conventional housing.[59] It is unclear if the contradictory findings among these studies are a result of animal sex, age, or differences between mouse strain (C57Bl/6 v. BALB/c, Table 2[59]).

Genetic background

Inbred mouse strains are commonly used for studying the effect of genetic background on bone phenotype.[60] Differences in the gut microbiota have been observed among inbred mouse strains,[61] raising the possibility that the microbiome may contribute to differences in bone phenotype among some of these mouse strains. The Toll-like receptor 5 (TLR5)-deficient mouse provides an example of how genetic background can alter the microbiome and organs distant from the gut. TLR5 is the innate immune receptor for bacterial flagellin and has no known endogenous ligand. Hence, changes in phenotype in the TLR5-deficient mouse are entirely dependent on host-microbe interactions. In the TLR5-deficient mouse, the inability to respond to flagellin leads to alterations in the gut microbiota, including reduced stability of the microbial community and increased expression of flagellin by commensal flora. Increased flagellin expression leads to increased bacterial motility and increased translocation of bacteria across the endothelial barrier where the bacteria can trigger an immune response leading to inflammation in the gut epithelium.[62] As a result of increased gut inflammation, the TLR5-deficient mouse develops mild insulin resistance, low-grade systemic inflammation, and mild increases in adiposity mimicking the condition of metabolic syndrome in humans.[63] Of interest, TLR5-deficient mice do not develop the metabolic syndrome-like traits if they are raised in a germ-free environment or have their gut flora decimated by chronic oral antibiotic treatment. Although the bone phenotype of the TLR5-deficient mouse has not been characterized, it is clear that loss of TLR5 can lead to alterations in the microbiome that have effects on distant organs, supporting the idea that some mice may have a microbiome-dependent bone phenotype.

Differential regulation of the microbiome among mouse strains may explain why germ-free BALB/c mice show reduced bone mass compared with conventionally raised animals,[59] whereas C57Bl/6 mice have increased bone mass when raised in a germ-free environment.[57] The potential role of the gut microbiome on bone is illustrated in the model depicted in Fig. 2. Consider a hypothetical mouse strain in which the genetic background leads to the development of a gut microbial community that results in reduced inflammation and promotes increased bone mass. Mice from this inbred strain, when raised in a germ-free environment, would display reduced bone mass compared with conventionally raised animals from the same strain. Alternatively, another mouse strain could develop a microbiome that enhances pro-inflammatory responses in the gut endothelium, leading to a reduced bone mass. Germ-free mice from this second strain would show increased bone mass compared with conventionally raised animals. The capacity of the gut microbiome to influence bone remodeling, therefore, has the potential to confound the interpretation of results in studies that use inbred mouse strains to understand the effect of genetic background on bone. For example, the bone phenotype in a mouse strain may be sensitive to factors that influence the microbiome (vendor, environment, etc.); secondly, it suggests that genetic characterization of phenotypes through quantitative trait locus (QTL) or related analyses[60] may identify bone phenotypes with genes related to regulation of the microbiome rather than genes that directly regulate bone physiology. In adult humans, bone mineral density is 50% to 80% heritable,[64] an association believed to be dominated by genetic background compared with environmental factors. The gut microbiome is also heritable,[65] and it is unclear as to how much heritability of bone mineral density (BMD) in humans is a result of heritability of gut flora.

Antibiotic treatment history

Administration of oral antibiotics represents an environmental factor that can greatly influence the gut microbiome. Recent studies have reported that antibiotic treatment can influence skeletal growth and bone mineral content in mice, as measured by whole-body DXA, and that the effects of antibiotics are influenced by sex and age. For example, low-dose antibiotics

Table 2. Summary of the Effects of Disruption or Absence of the Gut Flora on Bone Mass and Structure in Mice

Source	Mouse strain	Mouse age (weeks)	Mouse sex	Treatment	Bone measurement	Results
Sjogren et al.[57]	C57Bl/6J	7–9	Female	Germ-free	Micro-CT, pQCT, histomorphometry	Germ-free mice showed substantial increases in trabecular and cortical bone volume
Li et al.[58]	C57Bl/6J	20	Female	Germ-free	Micro-CT	Germ-free mice showed increased cortical thickness, no change in trabecular BV/TV
Schwarzer et al.[59]	BALB/c	7	Male	Germ-free	Micro-CT	Germ-free animals showed reduced cortical and trabecular bone
Cho et al.[66]	C57Bl/6J	7, 11	Female	Low-dose antibiotics	DXA	BMD increased at 7 weeks, no difference at 11 weeks
Cox et al.[67]	C57Bl/6J	20	Female	Low-dose antibiotics	DXA	BMD increased
Cox et al.[67]	C57Bl/6J	20	Male	Low-dose antibiotics	DXA	No change in BMD
Nobel et al.[68]	C57Bl/6J	3–20	Female	Pulsed oral antibiotics	DXA	BMC increased at 7 weeks of age, no difference at later ages

BV/TV = trabecular bone volume; BMD = bone mineral density; BMC = bone mineral content.
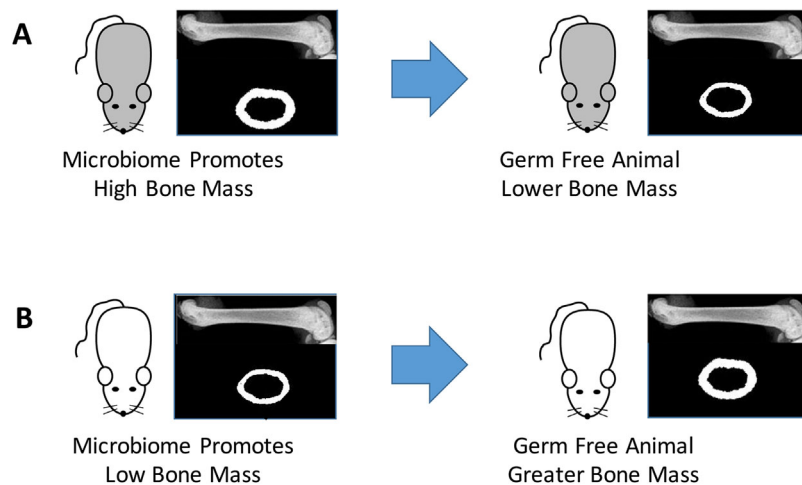
Fig. 2. Absence of gut flora may not have the same effect on bone in all experimental mouse models. (*A*) A mouse with a microbiome that supports a high bone mass phenotype. This mouse shows reduced bone strength when raised in a germ-free environment. (*B*) A mouse with a microbiome that promotes a low bone mass phenotype. This mouse shows increased bone mass when raised in a germ-free environment.

starting at weaning resulted in increased whole-body growth rates early in life.[66,67] Some changes in dual-energy X-ray absorptiometry (DXA)-derived bone mineral density were observed, but changes in BMD varied based on animal age and sex[66,67] (Table 2). In another study, pulsed antibiotic treatment (mimicking isolated rounds of treatment in children) followed by a high-fat diet resulted in increases in bone growth and whole-body bone mineral content.[68] In all cases, antibiotic treatment was associated with noticeable reductions in gut microbial diversity. These studies and others illustrate the important role of the gut microbiome in the regulation of bone homeostasis, particularly during the period of skeletal growth, and indicate the need for further investigation examining the relationship between the gut microbiome, genetic background, and bone.

Nutrition

The microbiome can have a profound effect on nutrient absorption and caloric uptake, which can have direct or indirect effects on bone metabolism. Chronic undernutrition has been shown to modify the gut microbiome and is associated with impaired bone growth during adolescence. In a recent study, microbiota from healthy and undernourished children (6 to 18 months of age) were transplanted into young germ-free mice. Five weeks after microbiota transplantation, mice receiving gut flora from healthy children saw more rapid increases in body weight and lean mass than those receiving microbiota from undernourished individuals.[69] Paradoxically, animals receiving gut flora from undernourished donors showed increased femoral cortical bone volume and bone mineral density compared with animals receiving microbiota from healthy donors. In a related study, using a different experimental design, animals raised on a nutrient-depleted diet showed impaired bone growth (reduced bone length) and the effect on bone growth was shown to be ameliorated to some degree by monocolonization with specific strains of *Lactobacillus* or complete reconstitution of the gut flora.[59] Although neither of these studies provided information on bone microstructure or biomechanics, they both clearly demonstrate that in cases of

nutritional deficiency, changes in the microbiota contribute independently to bone growth and development.

Sex hormones

Alterations in sex hormones are a primary stimulus for bone loss in humans, and recent investigations show that changes in the gut microbiome are correlated with alterations in hormone status and bone loss. Germ-free mice demonstrate resistance to bone loss after pharmacologically induced estrogen depletion.[58] These effects were attributed to failure to upregulate the production of pro-osteoclastogenic cytokines RANKL, tumor necrosis factor (TNF), and interleukin-17 that occurred in the estrogen-depleted mice grown under standard conditions. They then showed that treatment of mice grown under standard conditions with *Lactobacillus* or commercially available probiotic supplement were completely protected against bone loss associated with estrogen depletion.[58] Similarly, there are two reports that probiotic treatment prevented ovariectomy-induced bone loss in mice.[70,71] Two studies in rats suggest that treatment with antibiotics can ameliorate ovariectomy-induced bone loss.[72,73] Interestingly, inflammatory phenotypes associated with altered gut flora can differ between males and females,[63] suggesting that circulating sex hormones may influence microbiome-dependent phenotypes. In humans, the microbiome is altered during pregnancy,[74] providing further evidence that hormonal status influences the microbiome. Studies explaining sexual dimorphism in microbiome-dependent bone phenotypes have not been reported.

Probiotics and other clinical conditions

In addition to preventing estrogen depletion–induced bone loss, oral dosing with *Lactobacillus* probiotics has been associated within increased bone density in broader populations. Mature male mice (14 weeks old, C57Bl/6) treated with *Lactobacillus* probiotics for 4 weeks showed a 45% increase in femoral and vertebral trabecular bone volume fraction, increased bone formation, and a reduction in circulating pro-inflammatory cytokine expression, but no changes in cortical

bone were observed.[75] In contrast, females treated with the same probiotics exhibited no significant changes in bone phenotype or alterations in circulating inflammatory markers. The increases in bone volume fraction were attributed to alterations in calcium/and or nutrient absorption in the gut rather than alterations in systemic inflammation. Additionally, *Lactobacillus* probiotics were recently shown to prevent bone loss and alterations in adiposity and bone marrow fat in a model of type I diabetes (streptozotocin),[76] an effect attributed to maintenance of Wnt10b expression after induction of type I diabetes. A series of studies in rats suggest that prebiotics (molecules that promote growth of beneficial microbes) can mediate bone loss after ovariectomy (see Scholz-Ahrens and colleagues[77,78] for reviews) and can influence bone acquisition.[79,80]

Alterations in the gut microbiome have been observed in other clinical conditions in which osteopenia develops. For example, inflammatory bowel disease is associated with large changes in the gut microbiota,[81] and patients with inflammatory bowel disease are also at risk for osteopenia, osteoporosis, and associated fragility fractures[82,83] Osteopenia associated with inflammatory bowel disease has been attributed to impaired absorption of calcium, reduced circulating levels of vitamin D and vitamin K, or bone loss after treatment with glucocorticoids,[84] but recent studies have indicated that inflammation in the gut and systemically are associated with enhanced production of potent osteoclastogenic cytokines, which are key contributors to bone loss, independent of absorption of calcium and other nutrients.[83,85] Multiple animal models of colitis, including dextran sodium sulfate dosing, HLA-B27 transgenic rats, and IL10$^{-/-}$ knockout mice display reduced bone mass, bone volume fraction, and bone strength,[86–89] and the effects cannot be explained solely by impaired nutritional absorption.[84] Dextran sodium sulfate–induced colitis is enhanced in mice deficient in vitamin D receptor or an enzyme related to vitamin D hydroxylation (Cyp27B1)[90] and the gut microbiota is also changed, but whether these change in gut microbiota contribute to impaired bone mass or are simply correlated with alterations in vitamin D metabolism is not known.

Although existing evidence in mice clearly demonstrates that the microbiome can influence bone mass and structure, the specific mechanisms behind these changes are not well understood. Existing data are often conflicting, most likely because of differences in study design, including animal age and genetic background, as well as the imaging modalities employed (mouse DXA and/or inconsistent micro–computed tomography resolution). Many of the characteristics of bone phenotype that are well understood in mice and other animal models are not well understood in the context of the microbiome. For example, investigation of how the microbiome influences bone growth and development is limited to a few studies using mouse DXA. Relatively few of the studies reported to date have described the relative abundance of the commensal flora or reported correlations between the contents of the gut microbiota and bone phenotype.[59,69,70] None of the studies reported to date have described the effect of the gut microbiome on bone strength or tissue material properties.

Future Directions

Although the microbiome has been a topic of study since the advent of antibiotics, the development of high-throughput sequencing technologies over the last decade has allowed for rapid advancements in the field. These studies have established the importance of the microbiome in mammalian physiology, but the vast majority of the studies fail to provide insights into the mechanistic pathways responsible for these effects.[23]

Although the challenges of studying the effects of the microbiome on the major organ systems have been described,[91] the study of the effects of the microbiome on bone physiology presents special challenges. First, the relatively slow rate of change of bone presents a challenge because it is difficult to experimentally create a sustained change in gut flora. For example, a common approach for manipulating the gut flora is to transfer the microbiota from a donor into a germ-free animal. When exposed to the new host environment, the contents of the transferred microbiota change over time,[92] but the composition of the microbiota is rarely sustained long enough for a detectable change in skeletal phenotype (a month or more in mice). Second, preclinical studies relevant to osteoporosis concentrate on the adult skeletal phenotype, which requires older animals. Transfer of gut microbiota is not as effective in older animals[93,94] and the adult phenotype can be quite sensitive to the timing of microbial exposure,[95] making it difficult to study changes in gut flora after skeletal maturity. Lastly, methods of manipulating the gut flora as a form of treatment remain poorly understood. Methods of altering an established microbiome, or even replacing an "unhealthy" microbiome with a "healthy" microbiome, are still under development. Despite these challenges, there is substantial evidence that the microbiome has a significant effect on bone mass and bone physiology, and further studies are needed to not only define the mechanisms by which the microbiome modulates skeletal phenotype but also to develop approaches for manipulating the microbiome to maintain bone homeostasis and function.

We see two major challenges to advancing our understanding the links between the microbiome and bone. First, there have been few clinical reports linking the constituents of the gut microbiome to osteoporosis or other bone diseases. Correlations between bone mineral density and the gut microbiota have not yet been reported but, given the noninvasive nature of assessment, are feasible and could provide considerable insight. Second, given the conflicting effects of the gut microbiome on bone in mouse models, it is likely that the microbiome may be influencing bone phenotype and physiology in many well-established models. It may be necessary to repeat many well-established studies examining bone growth, mass, structure, strength, and fracture healing under conditions of altered or disrupted gut microbiota to understand the effects of the gut flora on bone physiology. In some cases, what we now consider to be an established effect of genetic background or a drug treatment may actually be secondary to regulation of the gut microbiota.

Disclosures

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FR0258

LGG and VSL#3 Probiotics Prevent Ovariectomy Induced Bone Loss and Induce Bone Anabolism in Normal Mice by Decreasing Gut Permeability and Inducing Wnt10b Production. <u>Jau-Yi Li*</u>[1], <u>Abdul Malik Tyagi</u>[1], <u>Emory Hsu</u>[1], <u>Marcelo Steiner</u>[1], <u>Jonathan Adams</u>[1], <u>Rheinallt Jones</u>[2], <u>Roberto Pacifici</u>[1]. [1]Emory University, School of Medicine, United states, [2]Department of Pediatrics, Emory University, United states

Probiotics, which are defined as viable microorganisms that confer a health benefit, prevent ovariectomy (ovx) induced bone loss, but their mechanism of action remains unknown. To investigate this matter, 10-week-old mice were ovx or sham operated and treated for 4 weeks with vehicle or the probiotics *L. rhamnosus* GG (LGG) and VSL#3 (1 X 10^{9} total bacteria twice weekly). Control treatments included vehicle, a strain of *E. coli* not exerting a probiotic effect, and LGG pili mutant (LGGM), a strain lacking epithelial adhesion. In a second experiment, LGG and VSL#3 were administered to intact 16 week old mice for 8 weeks. In vivo and in vitro µCT measurements revealed that LGG or VSL#3 completely prevented the increase in bone resorption and the loss of spinal and femoral bone volume (BV/TV) induced by ovx. LGGM was only partially effective and *E. Coli* did not prevent bone loss. Moreover, LGG or VSL#3 induced a significant increase in bone formation and BV/TV when fed to intact mice, while *E. Coli* and LGGM did not. These effects required the adherence of probiotics to the intestinal wall. We also found that ovx increases gut permeability by decreasing the gut epithelial tight junction proteins Claudin 2, 3, 15, and of the junction adhesion molecule Jam3. Increased gut permeability causes enhanced production of the inflammatory/osteoclastogenic factors RANKL, TNF and IL-17 in the intestine and the bone marrow. Treatment with LGG and VSL#3 (but not *E. Coli* and LGGM) normalized epithelial tight junction proteins thus preventing the detrimental effects of ovx on gut permeability and cytokine production in the gut and the bone marrow. Mechanistic studies of the bone anabolic activity of probiotics in intact mice revealed that LGG and VSL#3 increase stromal cell commitment to the osteoblastic lineage and osteoblast differentiation by activating Wnt signaling. This effect was secondary to increased production of the osteogenic Wnt ligand Wnt10b. In summary, administration of probiotics capable of adhering to the intestinal wall prevents the increase in gut permeability induced by estrogen deficiency, thereby dampening the ensuing osteoclastogenic immune cell response. In addition, in estrogen replete mice LGG and VSL#3 increase bone formation and bone mass by stimulating osteoblastogenesis via the Wnt10b/Wnt signaling pathway. Probiotics supplementation may thus represent an effective therapeutic strategy for the prevention and treatment of postmenopausal bone loss.

Disclosures: Jau-Yi Li, None.

FR0259

The Gut Microbiome Influences Bone Strength and Regulates Differences in Bone Biomechanical Phenotype Among Inbred Mouse Strains. <u>Jason Guss*</u>[1], <u>Michael Horsfield</u>[1], <u>Fernanda Fontenele</u>[1], <u>Taylor Sandoval</u>[1], <u>Marysol Luna</u>[1], <u>Fnu Apoorva</u>[1], <u>Svetlana Lima</u>[1], <u>Rodrigo Bicalho</u>[1], <u>Marjolein van der Meulen</u>[1], <u>Ankur Singh</u>[1], <u>Ruth Ley</u>[1], <u>Steven Goldring</u>[2], <u>Christopher Hernandez</u>[1]. [1]Cornell University, United states, [2]Hospital for Special Surgery, United states

The gut microbiome influence a number of conditions that alter bone structure and strength including obesity, diabetes, and inflammatory bowel disease. There are conflicting data regarding the effect of the microbiome on bone. Some studies suggest the absence of the microbiome increases bone mass [1] while others suggest that it decreases bone mass [2,3]. Here we test the idea that alterations in the gut microbiome modify bone mechanical properties by comparing the skeletal phenotype in wild type C57BL/6J mice and C57BL/6J mice deficient in toll-like receptor 5 (TLR5KO). TLR5 is an innate immune receptor for flagellin, and TLR5KO mice show an altered gut microbiome [4]. The effect of disruption of the microbiome on bone phenotype in each strain was determined by comparing animals treated with antibiotics from 4-16 weeks of age (1 g/L ampicillin, 0.5 g/L neomycin in drinking water) to untreated animals (n = 7-15/group, 39 animals total). Treatment shifted the gut microbiota composition from one dominated by the phylum Bacteroidetes to one dominated by Proteobacteria. Treatment was associated with reduced femoral bone strength in bending (Fig. 1). Reductions in strength caused by treatment could not be explained by alterations in bone morphology (differences between solid and dashed regression lines, p < 0.001), suggesting that treatment impaired bone tissue material properties. In untreated mice, there were small differences in strength between the WT and TLR5KO that could not be explained by bone morphology (solid regression lines differ, p < 0.01). Differences between mouse strains were eliminated by treatment (dashed lines similar). Femoral geometry did not differ among groups after accounting for body mass. Changes in bone biomechanical phenotype were correlated with alterations in splenic immune cell populations; TLR5KO mice had depleted B cell populations (p < 0.001), and antibiotic treated mice had reductions in both B and T cell populations (p < 0.001), suggesting that alterations in bone biomechanical phenotype may reflect modulation of the immune system by the gut flora. We conclude that changes in the gut microbiome can change bone mechanical properties by altering tissue material properties. Additionally, differences in bone biomechanical phenotype among mouse strains can depend on the presence and content of the gut microbiome. [1]Sjogren, K+, JBMR 2012 [2]Schepper, J+, ASBMR 2015 [3]Schwarzer, M+, Science 2016 [4]Vijay-Kumar, M+, Science 2010
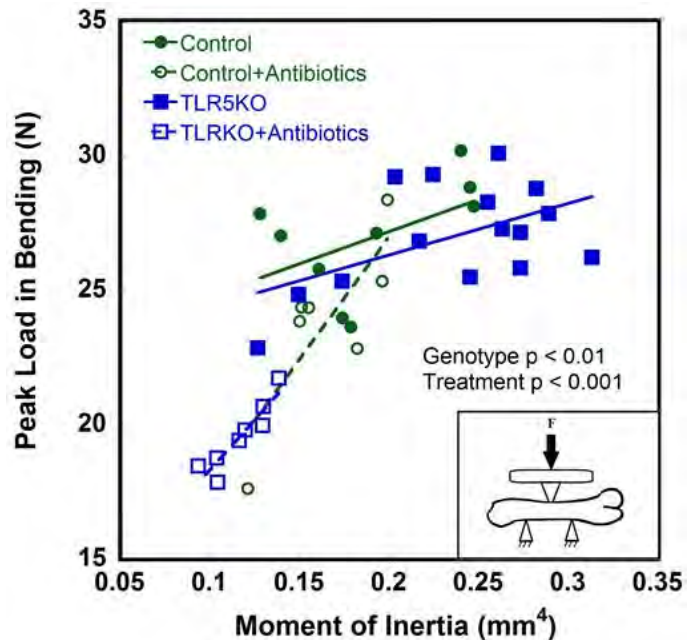
Moment of Inertia vs. Peak Load in Bending

Disclosures: Jason Guss, None.

FR0260

Delayed bone healing in type 1 diabetic rats is ameliorated by insulin treatment. <u>Ariane Zamarioli*</u>[1], <u>Francisco de Paula</u>[1], <u>Maysa Campos</u>[1], <u>Raquel Silva</u>[2], <u>José Volpon</u>[1]. [1]School of Medicine of Ribeirão Preto, Brazil, [2]School of Dentistry of Ribeirão Preto, Brazil

We assessed the effect of metabolic control on microstructural changes during fracture healing in diabetic rats. Thirty-eight female Wistar rats weighing approximately 200g were divided into three groups: (1) CON: weight-matched control rats, n=11; (2) DM: type 1 diabetic rats, n=13 and; (3) DM+INS: diabetic rats treated with insulin, n=14. DM and DM+INS rats received a single intravenous injection with streptozotocin to induce diabetes. Control rats were injected with citrate buffer alone. Diabetes was diagnosed based on blood glucose concentrations (≥250 mg/dL on two consecutive days). Rats from group DM+INS received daily insulin treatment to control blood glucose concentration below 200 mg/dL. Thirty days after diabetes induction (or buffer injection), the animals were anesthetized, and a closed bone fracture was produced in the right mid-femur. Then a surgical procedure with a 1-mm-diameter Kirschner wire was conducted for bone fragments stabilization. The status of the fracture was radiographically confirmed immediately after surgery and then followed-up weekly. Twelve rats either died or were excluded from the study (CON: 2 died during anaesthesia; DM: 2 were not diagnosed with diabetes and, 2 were excluded due to highly comminuted fracture; DM+INS: 2 were not diagnosed with diabetes, 2 were not successfully treated with insulin and, 2 died during anaesthesia). On day 14 post-surgery, twenty-six rats were killed, blood was collected for serum bone markers analysis and, the femurs were harvested in preparation for DXA assessment, µCT, and histological analysis. Bone callus was analyzed by calculating BMD and callus volume and by histological images. Poorly controlled glucose (DM rats without treatment) leads to dramatic changes in bone healing; with a deficit of 60% in callus volume and 40% in mineralization. Circulating level of IGF-1 was significantly reduced in these animals (-70%). On the other hand, the administration of insulin mitigates the deleterious effects of diabetes by accelerating bone callus formation not only in volume but also in bone density and ossification, which may be explained by an increase of 107% in circulating IGF-1 associated with a 47% reduction in circulating RANK-L. We concluded that poor diabetes control has detrimental effects on bone healing. However, insulin treatment not only improves the metabolic control, it restores the serum levels of IGF-I and RANK-L, creating condition for adequate fracture repair.